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RESEARCH ARTICLE

CHD1 acts via the *Hmgpi* pathway to regulate mouse early embryogenesis

Shinnosuke Suzuki¹, Yusuke Nozawa¹, Satoshi Tsukamoto², Takehito Kaneko³, Ichiro Manabe⁴, Hiroshi Imai¹ and Naojiro Minami^{1,*}

ABSTRACT

The protein CHD1 is a member of the family of ATPase-dependent chromatin remodeling factors. CHD1, which recognizes trimethylated histone H3 lysine 4, has been implicated in transcriptional activation in organisms ranging from yeast to humans. It is required for pre-mRNA maturation, maintenance of mouse embryonic stem cell pluripotency and rapid growth of the mouse epiblast. However, the function(s) of CHD1 in mouse preimplantation embryos has not yet been examined. Here, we show that loss of CHD1 function led to embryonic lethality after implantation. In mouse embryos in which *Chd1* was targeted by siRNA microinjection, the expression of the key regulators of cell fate specification *Pou5f1* (also known as *Oct4*), *Nanog* and *Cdx2* was dramatically decreased, starting at mid-preimplantation gene activation (MGA). Moreover, expression of *Hmgpi* and *Klf5*, which regulate *Pou5f1*, *Nanog* and *Cdx2*, was also significantly suppressed at zygotic gene activation (ZGA). Suppression of *Hmgpi* expression in *Chd1*-knockdown embryos continued until the blastocyst stage, whereas suppression of *Klf5* expression was relieved by the morula stage. Next, we rescued HMGPI expression via *Hmgpi* mRNA microinjection in *Chd1*-knockdown embryos. Consequently, *Pou5f1*, *Nanog* and *Cdx2* expression was restored at MGA and live offspring were recovered. These findings indicate that CHD1 plays important roles in mouse early embryogenesis via activation of *Hmgpi* at ZGA.

KEY WORDS: *Chd1*, Embryo development, Epigenetics, Zygotic gene activation, Mouse

INTRODUCTION

During preimplantation development in the mouse, the first important gene expression event is zygotic gene activation (ZGA), the first transcription from the newly formed zygotic genome, which occurs between the late 1-cell and 2-cell stages and is required for normal development (Hamatani et al., 2004; Levey et al., 1977; Li et al., 2010; Minami et al., 2007; Schultz, 1993; Wang and Dey, 2006; Warner and Versteegh, 1974). One-cell embryos treated with α -amanitin, an RNA polymerase II inhibitor, arrest development at the 2-cell stage because ZGA is suppressed (Levey et al., 1977; Warner and Versteegh, 1974). The second transcriptional event is mid-preimplantation gene activation (MGA), which occurs between the 4-cell and 8-cell stages. During this period, genes required for cell fate specification, such as the transcription factors *Pou5f1* (also known as *Oct4*), *Nanog*

and *Cdx2*, are expressed; these genes are key regulators governing formation of the inner cell mass (ICM) and trophectoderm (TE) (Hamatani et al., 2004; Nichols et al., 1998; Niwa et al., 2005; Strumpf et al., 2005; Wang and Dey, 2006; Yoshikawa et al., 2006). Thus, to understand cell fate specification, we must discern the regulatory mechanisms underlying expression of lineage-specific marker genes such as *Pou5f1*, *Nanog* and *Cdx2* at MGA. A previous study showed that a deficiency of *Tead4*, a transcription factor expressed during ZGA in mouse preimplantation embryos, led to a failure of cell fate specification due to suppression of *Cdx2* expression at MGA and developmental arrest at the morula stage (Yagi et al., 2007). Other experiments have also identified genes that are involved in cell fate specification by regulating the expression of *Pou5f1*, *Nanog* or *Cdx2* (Do et al., 2013; Elling et al., 2006; Home et al., 2012; Strumpf et al., 2005; Wang et al., 2010; Zhang et al., 2006, 2013).

During preimplantation development in mammals, dynamic changes occur in chromatin structure (Abdalla et al., 2009; Albert and Helin, 2010; Burton and Torres-Padilla, 2010; Corry et al., 2009; Morgan et al., 2005; Rasmussen and Corry, 2010; Shi and Wu, 2009). Previous studies showed that suppression of *Brg1* (*Smarca4* – Mouse Genome Informatics Database), a subclass of switch/sucrose non-fermentable (SWI/SNF) ATP-dependent chromatin remodelers, in mouse preimplantation embryos causes widespread expression of *Pou5f1* in the TE and leads to early embryonic death (Kidder et al., 2009; Wang et al., 2010). This suggests that chromatin-remodeling factors play important roles in mouse cell fate specification. CHD1 (chromodomain helicase DNA binding protein 1) belongs to the family of ATPase-dependent chromatin remodeling factors (Woodage et al., 1997). It recognizes the trimethylated lysine 4 of histone 3 (H3K4me3) (Sims et al., 2005) and has been implicated in transcriptional activation in yeast (Simic et al., 2003), *Drosophila* (Stokes et al., 1996) and mammalian cells (Sims et al., 2007). CHD1 is involved in pre-mRNA maturation (Sims et al., 2007), the maintenance of mouse embryonic stem cell (ESC) pluripotency (Gaspar-Maia et al., 2009), the maintenance of optimal transcriptional output in mouse ESCs and epiblast cells (Guzman-Ayala et al., 2015), and incorporation of the variant histone H3.3 into paternal pronuclear chromatin at fertilization in *Drosophila* embryos (Konev et al., 2007). However, the function(s) of CHD1 in mammalian preimplantation development is not fully understood.

Here, we investigated whether CHD1 functions during preimplantation development in the mouse. We observed that *Chd1* expression started to increase at the 2-cell stage, CHD1 was intensely localized in nuclei starting at the 2-cell stage and loss of CHD1 function by siRNA treatment led to embryonic lethality after implantation, due to suppression of *Pou5f1*, *Nanog* and *Cdx2* expression at MGA. Additionally, the expression of *Hmgpi* and *Klf5*, which regulate the expression of *Pou5f1*, *Nanog* and *Cdx2* during mouse preimplantation development (Ema et al., 2008; Lin et al., 2010; Yamada et al., 2010), was dramatically suppressed beginning at ZGA. However, *Hmgpi*

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mRNA microinjection in *Chd1*-knockdown embryos (*Chd1*-knockdown-*Hmgpi*-rescue embryos) rescued *Pou5f1*, *Nanog* and *Cdx2* expression and postimplantation embryo development. Based on these results, we propose that CHD1 has important roles in the development of pre- and postimplantation embryos via the activation of *Hmgpi* expression at ZGA.

RESULTS

Expression and localization of *Chd1* during preimplantation development in the mouse

To investigate the roles of CHD1 during preimplantation development in the mouse, *Chd1* expression and CHD1 localization were examined in preimplantation embryos (Fig. 1). *Chd1* mRNA

was expressed from the 1-cell to the blastocyst stage. Specifically, the expression increased at the 2-cell stage, peaked at the 8-cell stage and then dramatically decreased. CHD1 was localized in the nuclei of all blastomeres during preimplantation development and its staining intensity increased from the 2-cell stage onward.

Effects of *Chd1*-knockdown on the development of mouse embryos

Although the results of qRT-PCR and immunostaining revealed that the amounts of *Chd1* mRNA and protein were dramatically reduced after the 2-cell stage when si*Chd1* was injected at the 1-cell stage (Fig. 2),

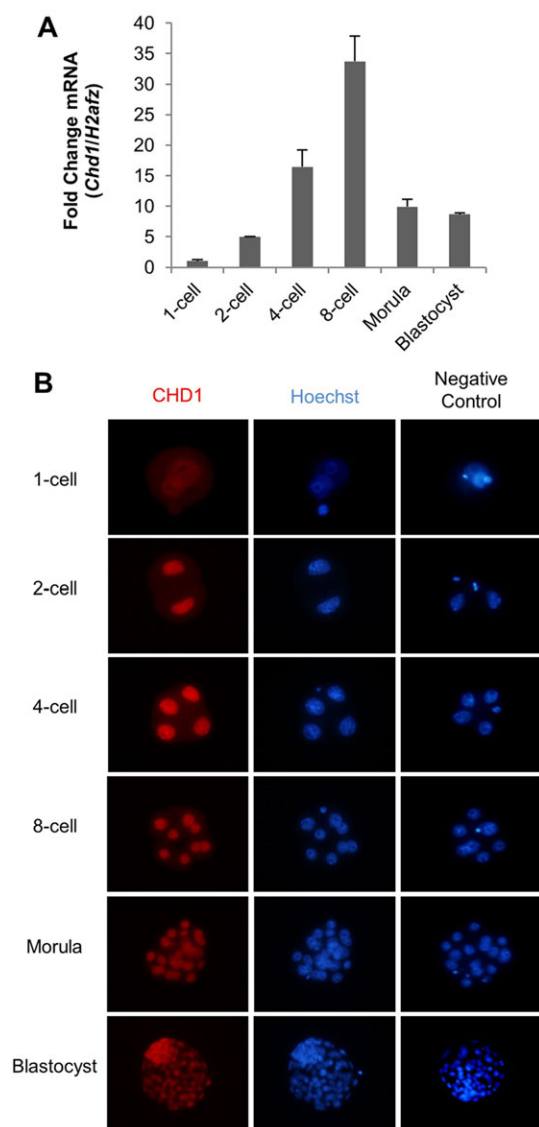


Fig. 1. *Chd1* expression and CHD1 localization during mouse preimplantation development. (A) qRT-PCR analysis of *Chd1* mRNA expression during mouse preimplantation development. Expression levels at each developmental stage were normalized to *H2afz*, used as internal control. The mRNA levels of the 1-cell embryos were defined as 1. Data are expressed as means \pm s.e.m. ($n=3$). (B) Localization of CHD1 during mouse preimplantation development (red, CHD1; blue, chromatin). Representative images of embryos at each stage are shown. Between 20 and 50 embryos were used in each experiment ($n=3$): in total, 103, 96, 117, 79, 81 and 124 embryos each for 1-cell, 2-cell, 4-cell, 8-cell, morula and blastocyst stage, respectively.

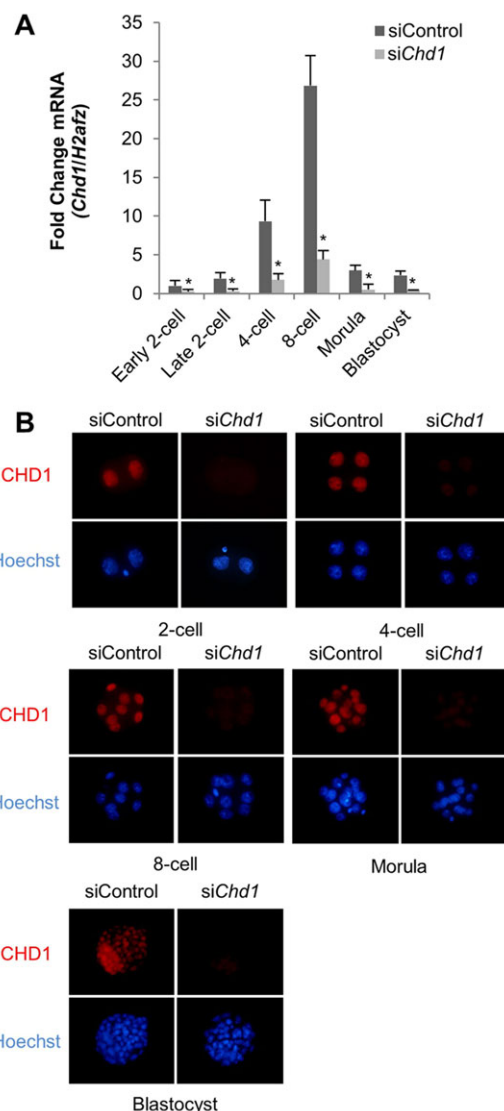


Fig. 2. Effects of *Chd1*-knockdown on expression of *Chd1* mRNA and protein in mouse preimplantation embryos. (A) qRT-PCR analysis of *Chd1* mRNA expression in control and *Chd1*-knockdown embryos from the early 2-cell to the blastocyst stage ($*P<0.05$). Expression levels were normalized to *H2afz*, used as internal control. The mRNA levels of the early 2-cell control embryos were defined as 1. Data are expressed as means \pm s.e.m. ($n=3$). (B) Immunofluorescence detection of CHD1 protein in control and *Chd1*-knockdown embryos (red, CHD1; blue, chromatin). Representative images of embryos at each stage are shown. Each image contains one embryo. Between 20 and 50 embryos were used in each experiment ($n=3$): in total, 96, 117, 79, 81 and 124 control embryos and 92, 121, 103, 144 and 137 *Chd1*-knockdown embryos each for late 2-cell, 4-cell, 8-cell, morula and blastocyst stage, respectively.

Chd1-knockdown embryos exhibited morphologically normal growth until the blastocyst stage, and *Chd1*-knockdown blastocysts (E4.5) had normal numbers of cells in both ICM and TE compared with controls (Fig. 3). In addition, there were no differences in hatching percentages between control and *Chd1*-knockdown embryos (Fig. 3). However, outgrowth experiments showed that the percentage of ICM-derived colony formation was dramatically decreased in *Chd1*-knockdown embryos (Fig. 3). Furthermore, the litter size after embryo transfer was significantly reduced in *Chd1*-knockdown embryos (Table 1).

CHD1 regulates zygotic expression of *Pou5f1*, *Nanog* and *Cdx2*

CHD1 is required for optimal transcriptional output in mouse ESCs (Guzman-Ayala et al., 2015). Therefore, to investigate the cause of litter size reduction in *Chd1*-knockdown embryos, we examined the effect of *Chd1*-knockdown on global gene expression in preimplantation embryos by BrUTP incorporation assay. The results showed that the reduction of global transcriptional activity does not occur in *Chd1*-knockdown embryos at the 2- and 4-cell stages (Fig. 4). To investigate the cause of litter size reduction in *Chd1*-knockdown embryos further, we assessed expression of the lineage-specific markers *Pou5f1*, *Nanog* and *Cdx2* at the mRNA and protein levels by qRT-PCR and immunostaining, respectively (Fig. 5). In control embryos, *Pou5f1* mRNA levels increased dramatically at the 4- and 8-cell stages, but

subsequently decreased. By contrast, *Pou5f1* mRNA expression was suppressed throughout these stages in *Chd1*-knockdown embryos. In control embryos, *Nanog* mRNA levels increased dramatically at the 4- and 8-cell stages, peaked at the morula stage and then decreased. By contrast, *Nanog* mRNA expression was suppressed throughout these stages in *Chd1*-knockdown embryos. With respect to *Cdx2*, in control embryos mRNA was first detected at the 8-cell stage and then gradually increased. Interestingly, *Cdx2* mRNA expression was suppressed during these stages in *Chd1*-knockdown embryos. Furthermore, in *Chd1*-knockdown embryos, immunofluorescence detection of OCT4 (encoded by *Pou5f1*), NANOG and CDX2 revealed that maternal OCT4 and NANOG proteins were maintained until the 4-cell stage, whereas for newly synthesized proteins, OCT4 and NANOG were reduced starting at the 8-cell stage, and CDX2 was reduced starting at the morula stage, respectively. The localization of OCT4 in the ICM, NANOG in the epiblast and CDX2 in the TE did not change in *Chd1*-knockdown embryos (Fig. 5).

CHD1 regulates the expression of *Hmgpi* and *Klf5* during preimplantation development

The mechanisms that regulate *Pou5f1*, *Nanog* and *Cdx2* expression during mouse preimplantation development have gradually been elucidated. However, the only factors identified to date that regulate the

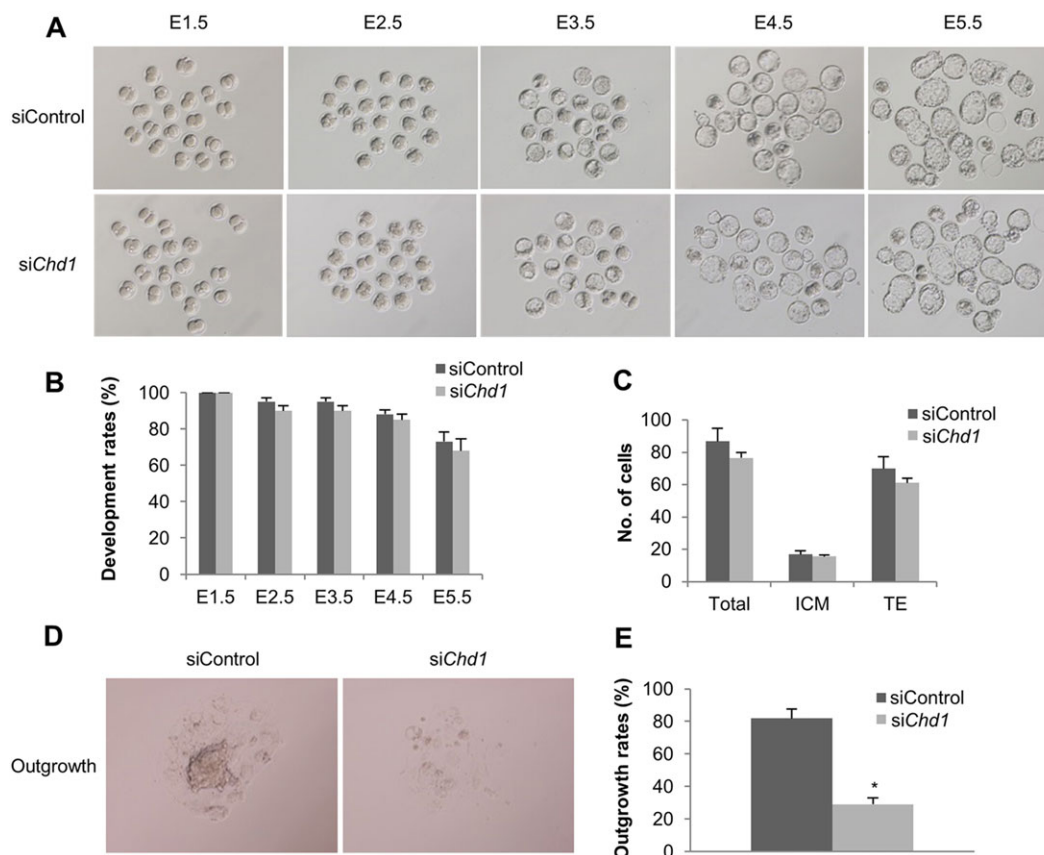


Fig. 3. Effect of *Chd1*-knockdown on the development of mouse embryos. (A) Pairs of representative images showing the development of preimplantation embryos injected with either siControl or siChd1. Embryos were photographed 36 h after *in vitro* fertilization and at 24-h intervals thereafter. (B) The percentages of normal development observed at E1.5 (≥ 2 -cell), E2.5 (≥ 4 -cell), E3.5 (morula), E4.5 (\geq blastocyst) and E5.5 (\geq hatching) in control and *Chd1*-knockdown embryos. Data are expressed as means \pm s.e.m. ($n=5$). Twenty embryos were used in each experiment: in total, 100 embryos each for the siControl and siChd1 arms. (C) The numbers of ICM and TE cells were assessed by counting OCT4⁺ cells and CDX2⁺ cells, respectively. Total numbers of cells were obtained by combining the numbers of ICM and TE cells. Data are expressed as means \pm s.e.m. ($n=20$). (D) Photographs depict representative results of outgrowth experiments for control and *Chd1*-knockdown embryos. (E) The successful percentage of ICM-derived colony formation in control and *Chd1*-knockdown embryos after 4 days in culture (* $P<0.05$). Data are expressed as means \pm s.e.m. ($n=5$). Between 25 and 120 embryos were used in each experiment: in total, 284 and 218 embryos for siControl and siChd1, respectively.

Table 1. Effect of *Chd1*-knockdown on development of mouse embryos

Treatment	Number of embryos transferred (number of recipient mice)	Number of live offspring (%)
siControl	45 (3)	22 (48.9)
siChd1	45 (3)	6 (13.3)*

* $P < 0.05$.

expression of *Pou5f1* and *Nanog* in ICM and *Cdx2* in TE are HMGPI and KLF5, both of which are transcription factors (Ema et al., 2008; Lin et al., 2010; Yamada et al., 2010). Therefore, we hypothesized that CHD1 regulates the expression of *Pou5f1* and *Nanog* in the ICM and *Cdx2* in the TE via activation of *Hmgpi* and *Klf5* expression. To investigate the effects of CHD1 on *Hmgpi* and *Klf5* expression, the levels of *Hmgpi* and *Klf5* mRNA and protein in *Chd1*-knockdown embryos were examined by qRT-PCR and immunostaining, respectively (Fig. 6). With respect to *Hmgpi*, in control embryos mRNA was first detected at the late 2-cell stage, peaked at the 4-cell stage and then gradually decreased. Conversely, *Hmgpi* mRNA expression was suppressed during these stages in *Chd1*-knockdown embryos. Immunofluorescent (IF) detection of HMGPI protein determined that its levels were also reduced from the 4-cell stage onward in *Chd1*-knockdown embryos (Fig. 6). With respect to *Klf5*, in control embryos mRNA was also first detected at the late 2-cell stage, peaked at the 8-cell stage and was maintained through the blastocyst stage. On the other hand, in *Chd1*-knockdown embryos, expression was suppressed at the 4- and 8-cell stages but recovered after the morula stage. IF detection of KLF5 demonstrated that the amount of KLF5 protein was remarkably reduced at the 4- and 8-cell stages; however, KLF5 protein levels gradually recovered after the morula stage in *Chd1*-knockdown embryos (Fig. 6).

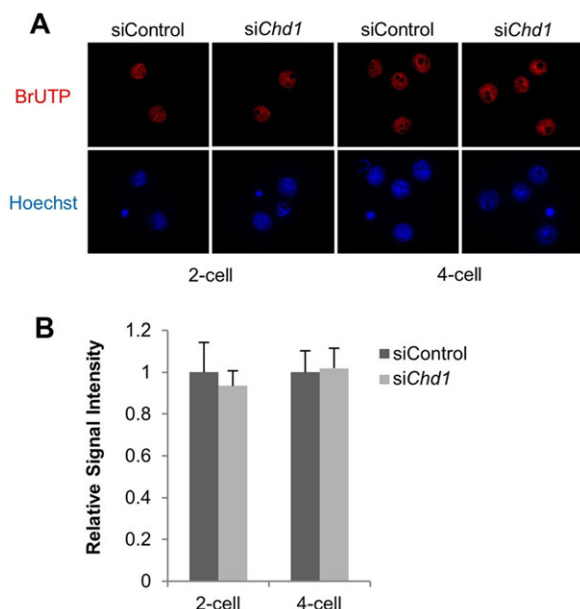


Fig. 4. Effects of *Chd1*-knockdown on global gene expression.

(A) Immunofluorescence detection of BrUTP incorporation as a measure of genome-wide transcription in control and *Chd1*-knockdown embryos (red, BrUTP; blue, chromatin). Twenty embryos were used in each experiment ($n=3$); in total, 60 embryos each for siControl and siChd1. (B) Quantitation of fluorescence signal intensity for BrUTP incorporation assay. The signal intensity of BrUTP in the nuclei was individually quantitated. The signal intensity of the nuclei in control embryos was defined as 1. Data are expressed as means \pm s.e.m. ($n=15$).

HMGPI rescue can recover outgrowth and litter size in *Chd1*-knockdown embryos

To investigate whether *Hmgpi* regulates the expression of *Pou5f1*, *Nanog* and *Cdx2* under the control of CHD1, we rescued HMGPI expression by injecting *Hmgpi* mRNA into *Chd1*-knockdown embryos. Outgrowth and embryo transfer experiments showed that normal ICM-derived colony formation and litter size were recovered in *Chd1*-knockdown-*Hmgpi*-rescue embryos (Fig. 7, Table 2). qRT-PCR results indicated that *Chd1* mRNA levels were not restored in *Chd1*-knockdown-*Hmgpi*-rescue embryos at any stage (Fig. 7). Nonetheless, qRT-PCR and immunostaining results showed that *Pou5f1* and *Nanog* mRNA levels recovered, starting at the 4-cell stage onward, and that OCT4 and NANOG protein expression levels recovered starting at the 8-cell stage onward (because maternal OCT4 and NANOG proteins remain until the 4-cell stage in *Chd1*-knockdown-*Hmgpi*-rescue embryos). Furthermore, *Cdx2* mRNA levels were restored starting at the 8-cell stage onward, and CDX2 protein levels were recovered starting at the morula stage onward in *Chd1*-knockdown-*Hmgpi*-rescue embryos (Fig. 7). Meanwhile, qRT-PCR and immunostaining of *Klf5* revealed that HMGPI rescue had no effect on the expression of *Klf5* mRNA or protein (see supplementary material Fig. S1).

DISCUSSION

CHD1, a chromatin remodeling factor, recognizes H3K4me3 (Sims et al., 2005), facilitates pre-mRNA maturation (Sims et al., 2007) and is required for the maintenance of mouse ESC pluripotency (Fazio and Panning, 2010; Gaspar-Maia et al., 2009). CHD1 is also necessary at fertilization in *Drosophila* embryos for the incorporation of the variant histone H3.3 into paternal pronuclear chromatin in the absence of transcription (Konev et al., 2007). Recent work showed that *Chd1*-knockout embryos undergo developmental arrest at E6.5 due to a failure to maintain the epiblast (Guzman-Ayala et al., 2015). However, because *Chd1*-knockout embryos were produced from heterozygous intercrosses, maternal *Chd1* mRNA and protein are not suppressed at ZGA in *Chd1*-knockout embryos. Here, we showed that in *Chd1*-knockdown embryos, expression of *Chd1* mRNA and proteins were suppressed at ZGA, and *Chd1* knockdown led to embryonic lethality after implantation. Additionally, we showed that suppression of zygotic *Pou5f1*, *Nanog* and *Cdx2* expression continued through the blastocyst stage via suppression of *Hmgpi*. However, we also showed that CHD1 was not required for global transcriptional activity at ZGA and MGA, suggesting that CHD1 is involved in selective regulation of gene expression that governs normal embryogenesis and the maintenance of pluripotency during mouse preimplantation development.

The expression of *Pou5f1*, *Nanog* and *Cdx2* at MGA is regulated by *Hmgpi* and *Klf5*, both of which begin to be expressed at ZGA (Ema et al., 2008; Lin et al., 2010; Yamada et al., 2010). In embryos lacking functional *Hmgpi* or *Klf5*, developmental arrest occurs during peri-implantation development due to suppression of *Pou5f1*, *Nanog* and *Cdx2* expression (Ema et al., 2008; Lin et al., 2010; Yamada et al., 2010), suggesting that *Hmgpi* and *Klf5* regulate the initiation of cell fate specification. However, the detailed mechanisms underlying regulation of *Pou5f1*, *Nanog* and *Cdx2* by HMGPI and KLF5 are unknown. The results of this study showed that in *Chd1*-knockdown embryos, suppression of *Hmgpi* expression continued until the blastocyst stage, whereas suppression of *Klf5* expression was relieved by the morula stage, when *Klf5* expression recovered spontaneously. Accordingly, we hypothesized that suppression of *Hmgpi* through the blastocyst accounts for

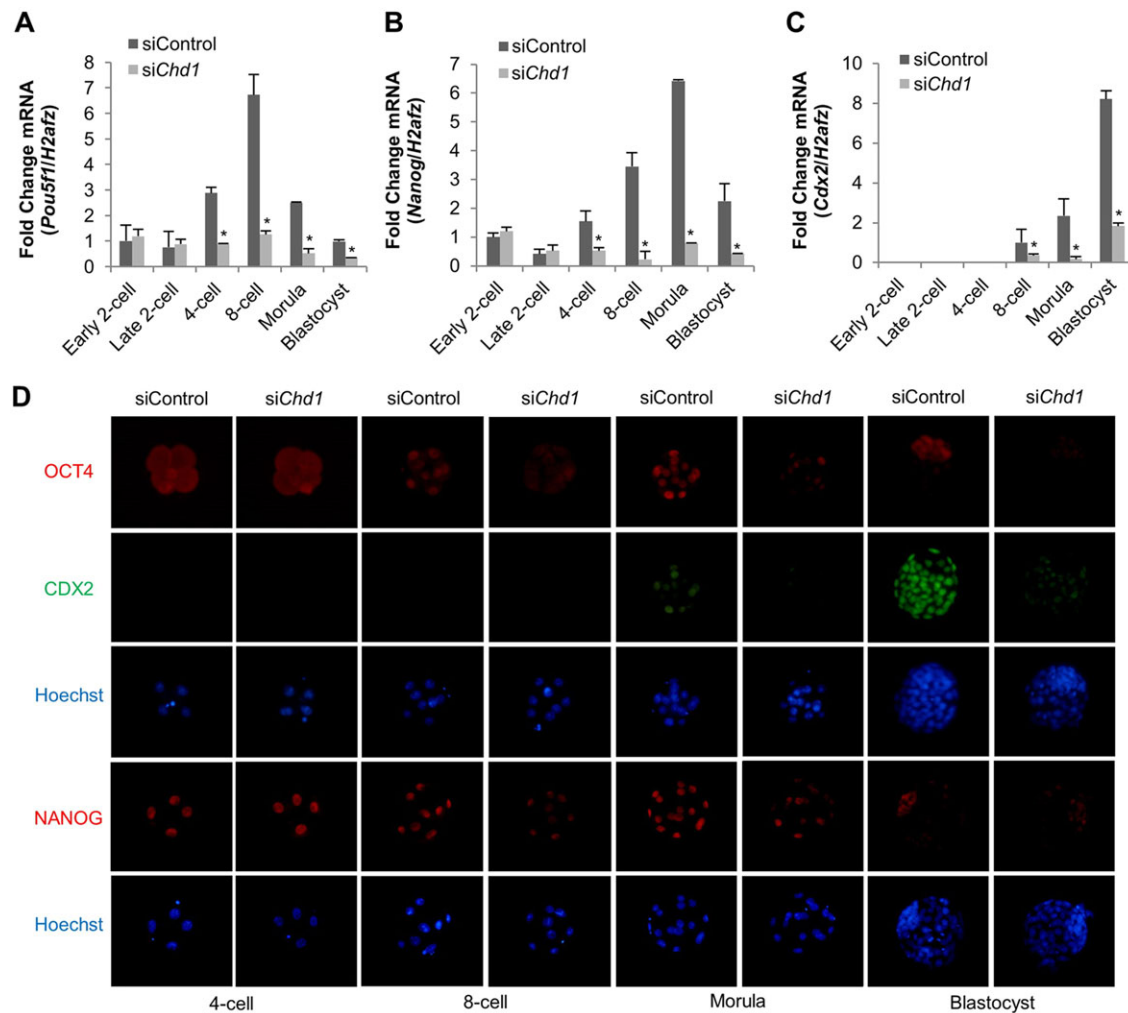


Fig. 5. CHD1 is required for development of preimplantation embryos. qRT-PCR analysis of (A) *Oct4*, (B) *Nanog* and (C) *Cdx2* mRNA in control and *Chd1*-knockdown embryos from the early 2-cell to the blastocyst stages (* $P < 0.05$). Expression levels were normalized to *H2afz*, used as internal control. mRNA levels of the early 2-cell control embryos were defined as 1 for *Oct4* and *Nanog* quantitation, and mRNA levels of the 8-cell control embryos were defined as 1 for *Cdx2* quantitation. Data are expressed as means \pm s.e.m. ($n = 3$). (D) Immunofluorescence detection of OCT4, NANOG and CDX2 in control and *Chd1*-knockdown embryos (red, OCT4; red, NANOG; green, CDX2; blue, chromatin). Representative images of embryos at each stage are shown. Between 20 and 50 embryos were used in each experiment (OCT4 and CDX2 double-staining; $n = 3$): in total, 122, 87, 88 and 134 control embryos and 97, 106, 142 and 178 *Chd1*-knockdown embryos each for 4-cell, 8-cell, morula and blastocyst stage, respectively. Between 20 and 30 embryos were used in each experiment (NANOG; $n = 3$): in total, 65, 60, 85 and 88 control embryos and 74, 68, 90 and 89 *Chd1*-knockdown embryos each for 4-cell, 8-cell, morula and blastocyst stage, respectively.

suppression of *Pou5f1*, *Nanog* and *Cdx2* expression, normal ICM-derived colony formation and the numbers of live offspring. Thus, we investigated the effects of *Hmgpi*-rescue in *Chd1*-knockdown embryos on *Pou5f1*, *Nanog* and *Cdx2* expression and postimplantation development. We observed that *Pou5f1*, *Nanog* and *Cdx2* expression, normal ICM-derived colony formation and the numbers of live offspring were all restored in *Chd1*-knockdown-*Hmgpi*-rescue embryos, suggesting that CHD1 plays important roles as a trigger for *Pou5f1*, *Nanog* and *Cdx2* expression through the regulation of *Hmgpi* expression at ZGA. Furthermore, we showed that the rescue of HMGPI had no effect on *Klf5* expression in *Chd1*-knockdown-*Hmgpi*-rescue embryos, suggesting that there is no direct interaction between *Hmgpi* and *Klf5* during preimplantation development.

The maintenance of pluripotency depends on OCT4 and NANOG functions during preimplantation development (Chen et al., 2009; Mitsui et al., 2003; Nichols et al., 1998; Shao et al., 2008). *Pou5f1* and *Nanog*, both of which are ICM markers,

negatively interact with *Cdx2*, a TE marker, and these three genes are key regulators in cell fate specification (Chen et al., 2009; Niwa et al., 2005; Ralston et al., 2010; Strumpf et al., 2005). *Pou5f1* or *Nanog* knockout mouse embryos can develop into morphologically normal blastocysts; however, developmental arrest occurs during the postimplantation period due to a loss of pluripotency (Mitsui et al., 2003; Nichols et al., 1998; Ralston et al., 2010). By contrast, *Cdx2* knockout mouse embryos are arrested at the early blastocyst stage because they fail to maintain the blastocoel (Strumpf et al., 2005). Therefore, it is possible that suppression of *Pou5f1*, *Nanog* and *Cdx2* could account for the exhibition of embryonic lethality. Although *Chd1*-knockdown embryos can develop into morphologically normal blastocysts despite suppression of *Cdx2*, it is probable that low levels of remaining CDX2 protein might function during TE development. Guzman-Ayala et al. (2015) reported that CHD1 is required for the maintenance of the epiblast at E5.5 (Guzman-Ayala et al., 2015); however, our results show that CHD1 is required for the expression of *Hmgpi* at ZGA and that

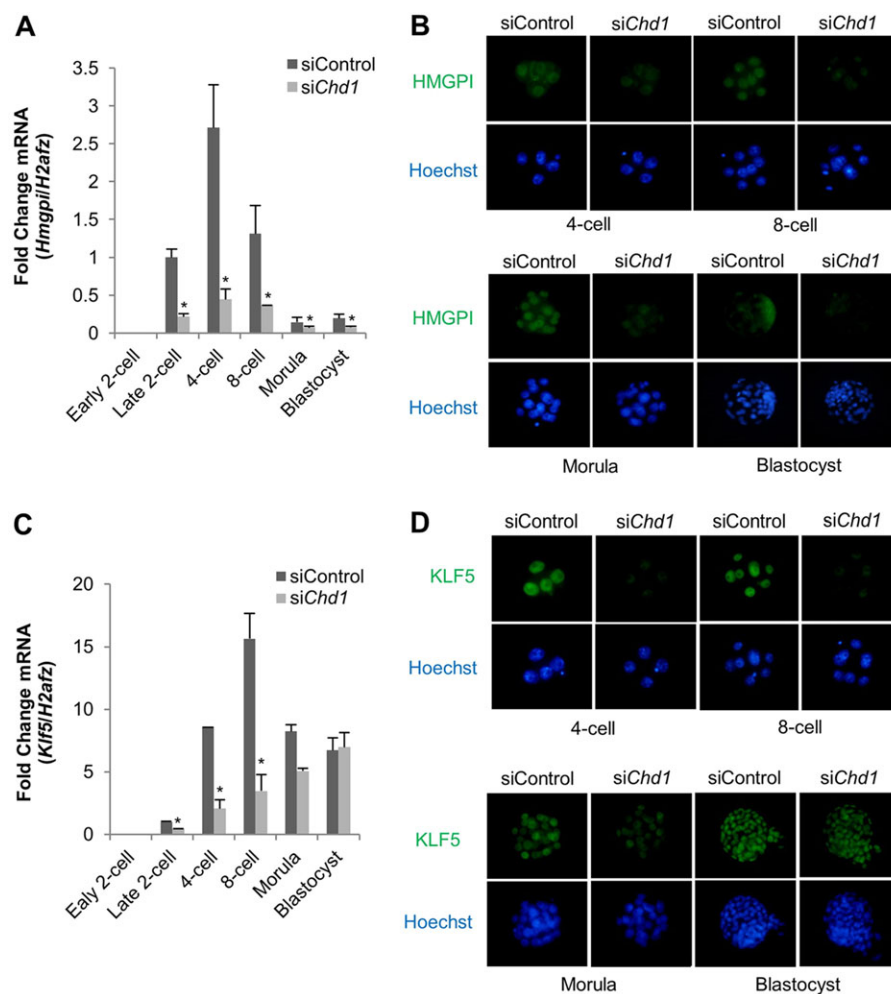


Fig. 6. Effects of *Chd1*-knockdown on *Hmgpi* and *Klf5* expression. qRT-PCR analyses of (A) *Hmgpi* and (C) *Klf5* mRNA in control and *Chd1*-knockdown embryos from the early 2-cell to the blastocyst stages (**P* < 0.05). Expression levels were normalized to *H2afz*, used as an internal control. The mRNA levels of the late 2-cell control embryos were defined as 1. Data are expressed as means \pm s.e.m. (*n* = 3). (B) Immunofluorescence detection of HMGPI in control and *Chd1*-knockdown embryos (green, HMGPI; blue, chromatin). Representative images of embryos at each stage are shown. Between 20 and 30 embryos were used in each experiment (*n* = 3): in total, 59, 75, 95 and 102 control embryos, and 76, 82, 75 and 114 *Chd1*-knockdown embryos each for 4-cell, 8-cell, morula and blastocyst stage, respectively. (D) Immunofluorescence detection of KLF5 in control and *Chd1*-knockdown embryos (green, KLF5; blue, chromatin). Representative images of embryos at each stage are shown. Between 20 and 50 embryos were used in each experiment (*n* = 3): in total, 87, 59, 117 and 126 control embryos, and 90, 76, 122 and 144 *Chd1*-knockdown embryos each for late 2-cell, 4-cell, 8-cell, morula and blastocyst stage, respectively.

global transcriptional activity is not changed at ZGA and MGA in *Chd1*-knockdown embryos. These results suggest that CHD1 selectively regulates transcription during mouse early preimplantation development. Together, these results suggest that CHD1 plays dual roles during embryogenesis: (1) resulting in *Hmgpi* and *Klf5* expressions at ZGA during preimplantation development, and (2) acting as a regulator of global gene expression after implantation.

Developmentally important genes are marked by H3K4me3 at pre-ZGA and have a strong propensity to be activated after ZGA (Lindeman et al., 2011). In this study, we demonstrated that CHD1, a chromatin-remodeling factor that recognizes H3K4me3, is involved in the regulation of gene expression at ZGA and affects the development of mouse pre- and post-implantation embryos. Therefore, to understand the regulatory mechanisms of early embryogenesis, it is important to discern the transcriptional mechanisms in which H3K4me3 is involved at ZGA.

In conclusion, we demonstrated that CHD1 regulates the initiation of zygotic *Oct4*, *Nanog* and *Cdx2* expression at MGA via activation of *Hmgpi* and *Klf5* expression at ZGA. Thereafter, both HMGPI and KLF5, under the control of CHD1, regulate the expression of *Pou5f1*, *Nanog* and *Cdx2*, and thereby control initiation of cell fate specification. Subsequently, *Klf5* expression escapes from the control of CHD1, and HMGPI regulates the expression of *Pou5f1* and *Nanog* in the future ICM/EPI and the expression of *Cdx2* in the future TE.

MATERIALS AND METHODS

Superovulation, embryo collection and embryo culture

Eight- to ten-week-old ICR female mice (Japan SLC) were superovulated by injecting 5 IU of equine chorionic gonadotropin (eCG; ASUKA), followed by 5 IU of human chorionic gonadotropin (hCG; ASUKA) 48 h later. Unfertilized eggs were harvested 14 h after the hCG injection and placed in a 90- μ l droplet of HTF supplemented with 4 mg/ml BSA (A3311; Sigma-Aldrich) (Minami et al., 2001). Spermatozoa were collected from the cauda epididymis of 11- to 15-week-old ICR male mice (Japan SLC) and cultured for 2 h in 100- μ l of HTF supplemented with 4 mg/ml BSA. After preincubation, sperm were introduced into fertilization droplets at a final concentration of 1×10^6 cells/ml. After a 3-h incubation, fertilized 1-cell embryos were collected and washed three times in KSOM supplemented with amino acids (Ho et al., 1995) and 4 mg/ml BSA, and then were either used for microinjection (39) or cultured until embryonic day 4.5 (E4.5) in the same medium under mineral oil (Sigma-Aldrich) at 37°C in an atmosphere of 5% CO₂.

Chd1 siRNA injection

Approximately 5–10 μ l of 100 μ M *Chd1* siRNA (si*Chd1*) (RNAi, Japan; 5'-GGUUUACUUAGGCGACAUUAA-3') or the control scrambled sequence siRNA (siControl; RNAi) (5'-GCGUUUUAUAGCAUUAUGCGAA-3') in annealing buffer [consisting of 30 mM HEPES-KOH (pH 7.4), 100 mM KOAc and 2 mM Mg(OAc)₂] was microinjected into the cytoplasm of 1-cell embryos between 3 and 4 h after insemination. After injection, the embryos were cultured in KSOM medium supplemented with amino acids (Ho et al., 1995) with 4 mg/ml BSA under mineral oil (Sigma-Aldrich) at 37°C in an atmosphere of 5% CO₂. To examine developmental competency and hatching ability, embryos were observed at 36 (E1.5), 60 (E2.5), 84 (E3.5),

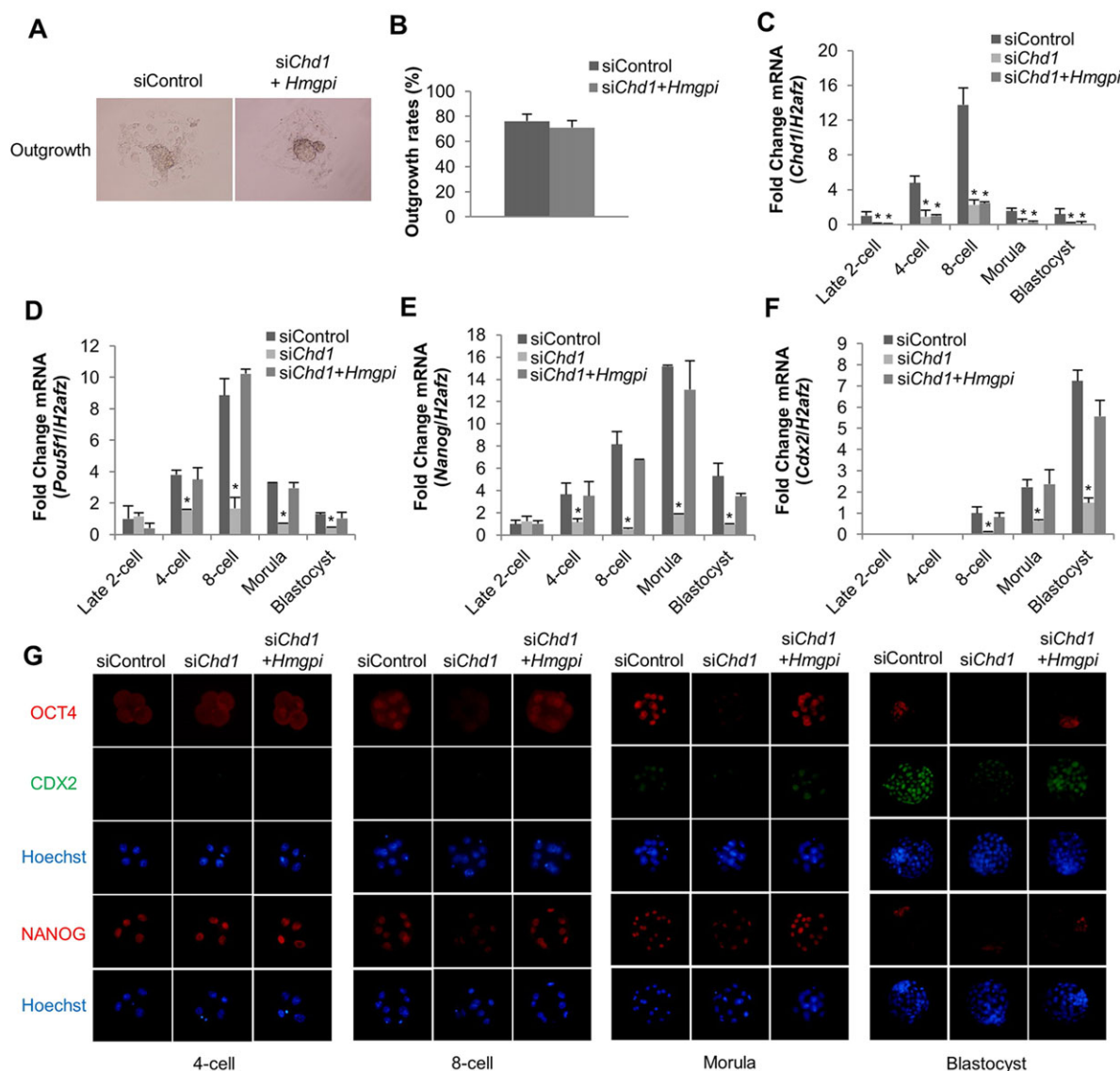


Fig. 7. Effects of *Hmgpi*-rescue in *Chd1*-knockdown embryos on development of pre- and post-implantation embryos. (A) Photographs depict representative results of outgrowth experiments from control and *Chd1*-knockdown-*Hmgpi*-rescue embryos. (B) Percentage of ICM-derived colony formation in control and *Chd1*-knockdown-*Hmgpi*-rescue embryos after 4 days in culture (* $P < 0.05$). Data are expressed as means \pm s.e.m. ($n = 3$). Between 20 and 80 embryos were used in each experiment: in total, 172 and 129 embryos for siControl and siChd1 + *Hmgpi* mRNA, respectively. qRT-PCR analysis of (C) *Chd1*, (D) *Pou5f1*, (E) *Nanog* and (F) *Cdx2* mRNA in control, *Chd1*-knockdown and *Chd1*-knockdown-*Hmgpi*-rescue embryos from the late 2-cell to the blastocyst stages. Expression levels were normalized to *H2afz*, used as internal control. The mRNA levels of the late 2-cell control embryos were defined as 1 for *Chd1*, *Pou5f1* and *Nanog* quantitation, and mRNA levels of the 8-cell control embryos were defined as 1 for *Cdx2* quantitation. Data are expressed as means \pm s.e.m. ($n = 3$). (G) Immunofluorescence detection of OCT4, NANOG and CDX2 in control, *Chd1*-knockdown and *Chd1*-knockdown-*Hmgpi*-rescue embryos (red, OCT4; red, NANOG; green, CDX2; blue, chromatin). Representative images of embryos at each stage are shown. Between 20 and 50 embryos were used in each experiment (OCT4 and CDX2 double-staining; $n = 3$): in total, 122, 87, 88 and 134 control embryos, 97, 106, 142 and 178 *Chd1*-knockdown embryos, and 93, 85, 137 and 155 *Chd1*-knockdown-*Hmgpi*-rescue embryos each for 4-cell, 8-cell, morula and blastocyst stage, respectively. Between 20 and 30 embryos were used in each experiment (NANOG; $n = 3$): in total, 60, 64, 82 and 81 control embryos, 66, 63, 79 and 77 *Chd1*-knockdown embryos, and 68, 62, 72 and 68 *Chd1*-knockdown-*Hmgpi*-rescue embryos each for 4-cell, 8-cell, morula and blastocyst stage, respectively.

108 (E4.5) and 132 h (E5.5) after insemination. Furthermore, the embryos were harvested for quantitative RT-PCR (qRT-PCR), IF staining, outgrowth analysis or embryo transfer.

RNA extraction and qRT-PCR

Embryos were harvested after culturing for 14, 20, 36, 48, 55, 76 and 108 h after insemination, when most of the oocytes had reached the 1-cell, early 2-cell, late 2-cell, 4-cell, 8-cell, morula and blastocyst stages, respectively. RNA extraction and qRT-PCR were performed as described previously (Suzuki et al., 2013). Total RNA from 30 embryos was extracted using TRIzol (Invitrogen). Transcription levels were determined on three different

sets of 30 embryos per stage and normalized to *H2afz*, a stable reference gene used for normalization of gene expression in mouse preimplantation embryos (Jeong et al., 2005; Mamo et al., 2007); relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primers used for qPCR are listed in supplementary material Table S1.

IF staining

Embryos for immunostaining were collected as described above. For staining of CHD1, HMGPI and KLF5, zona pellucida was removed from the embryo by acid Tyrode's solution (pH 2.5) and the embryos were fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde (Sigma-Aldrich) for

Table 2. Effect of *Hmgpi*-rescue on development of *Chd1*-knockdown embryos

Treatment	Number of embryos transferred (number of recipient mice)	Number of live offspring (%)
siControl	45 (3)	20 (44.4)
si <i>Chd1</i> + <i>Hmgpi</i> mRNA	45 (3)	22 (48.9)

20 min at 4°C. After washing three times in PBS containing 0.3% polyvinylpyrrolidone (PVP K-30, Nacalai Tesque; PBS/PVP), embryos were treated with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 40 min at room temperature (RT), blocked in PBS containing 1.0% BSA (A9647, Sigma-Aldrich) for 1 h at RT (for CHD1 and KLF5) or 3.0% BSA overnight at 4°C (for HMGPI) and then incubated overnight at 4°C with a rabbit anti-CHD1 antibody (1:25 dilution; #4351, Cell Signaling) or a rat anti-KLF5 antibody (1:500 dilution; Shindo et al., 2002) or for 1 h at RT with a rabbit anti-HMGPI antibody (1:100 dilution; Yamada et al., 2010) in PBS containing 1.0% BSA (PBS/BSA). After washing with PBS/BSA, embryos were incubated in PBS/BSA containing a secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG, 1:250 dilution, Invitrogen, R37117; Alexa Fluor 488 goat anti-rat IgG, 1:300 dilution, Invitrogen, A-11006; or Alexa Fluor 488 goat anti-rabbit IgG, 1:500 dilution, Invitrogen, A27034) for 1 h at RT. After washing three times in PBS/BSA for 15 min each, nuclei were stained in PBS/BSA containing 10 µg/ml Hoechst 33342 (Sigma-Aldrich) for 10 min. Immunostaining with normal rabbit IgG (sc-2027, Santa Cruz; 1:200 dilution) was included as a negative control for the specificity of the anti-CHD1 antibody. For double-staining of OCT4 and CDX2, immunofluorescence staining was performed as previously described (Isaji et al., 2013). For NANOG staining, embryos were fixed in PBS containing 4% paraformaldehyde for 10 min at RT. After three washes in PBS/PVP, fixed embryos were treated with 0.5% Triton X-100 (Sigma-Aldrich) in PBS for 30 min at RT, blocked in PBS containing 10% fetal bovine serum and 0.1% Triton X-100 (PBS/FBS) for 1 h at RT. Next, embryos were incubated overnight at 4°C with a rabbit anti-NANOG antibody (1:1000 dilution, 1 µg/ml; ab5731, Millipore) in PBS/FBS. Embryos were washed three times in PBS/FBS and then incubated for 1 h at RT with the appropriate secondary antibody diluted 1:750 (Alexa Fluor 594-conjugated goat anti-rabbit IgG, Invitrogen, A-11037). After staining, the samples were washed three times in PBS/BSA or PBS/FBS for 15 min, and nuclei were stained for 10 min at RT in PBS containing 10 µg/ml Hoechst 33342 (Sigma-Aldrich). For staining of BrUTP, immunofluorescence staining was performed as described previously (Aoki et al., 1997). After IF staining, embryos were mounted on slides in 50% glycerol/PBS and fluorescent signals were detected with a fluorescence microscope (BX50, Olympus). At least 30 oocytes were examined in each group. The numbers of ICM and TE cells were determined by counting OCT4⁺ and CDX2⁺ cells, respectively. Total embryonic cell numbers were obtained by adding the numbers determined for the ICM and TE cells. To count the cell number and observe the localization of OCT4, NANOG and CDX2 in *Chd1*-knockdown embryos, exposure time was extended relative to that used for control embryos.

Outgrowth analysis

Outgrowth analysis was carried out on E3.5 embryos after removal of the zona pellucida by acid Tyrode's solution (pH 2.5). The embryos were cultured in ES medium (Glasgow modification of Eagle's medium/GMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate (Sigma), 0.1 mM MEM non-essential amino acid (Invitrogen), 0.1 mM 2-mercaptoethanol (Wako), 0.1 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) on a 0.1% gelatin-coated dish (Sigma-Aldrich) at 37°C in an atmosphere of 5% CO₂. After 4 days in culture, the embryos were photographed and the percentage of blastocysts that had undergone outgrowth was calculated.

Embryo transfer

Fifteen embryos that reached the 2-cell stage after microinjection were transferred into the oviducts of surrogate females (Japan SLC), which had

been mated with vasectomized males the day before embryo transfer. These females were sacrificed at day 19 and pups were counted. The experiment was repeated three times.

BrUTP incorporation assay

Embryos were harvested after culturing for 36 and 48 h after insemination, when most of the oocytes reached the late 2- and 4-cell stages, respectively. BrUTP incorporation assay was performed by electroporation using the Super Electroporator NEPA 21 (NEPAGENE). Embryos were washed twice in PBS and then transferred in a line on the glass chamber between metal plates filled with PBS containing 10 mM BrUTP (Sigma-Aldrich). The poring pulse (voltage: 225 V, pulse length: 0.5 ms, pulse interval: 50 ms, number of pulses: 4, +) and the transfer pulse (voltage: 20 V, pulse length: 50 ms, pulse interval: 50 ms, number of pulses: 5, ±) were selected. The embryos were washed three times and cultured in KSOM supplemented with amino acids (Ho et al., 1995) and 4 mg/ml BSA under mineral oil (Sigma-Aldrich) at 37°C in an atmosphere of 5% CO₂ for 1 h. Subsequently, the embryos were treated with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 3 min at RT after removal of the zona pellucida by acid Tyrode's solution (pH 2.5) and fixed in PBS containing 4% paraformaldehyde (Sigma-Aldrich) overnight at 4°C. After washing three times in PBS/PVP, they were subjected to immunostaining. BrUTP-related signals were observed using a fluorescence microscope (BZ-X700, Keyence), and the fluorescence signal intensity was quantitated by using measurement module BZ-H3C (Hybrid Cell Count, Keyence).

In vitro transcription and microinjection

The *Hmgpi* ORF was generated by PCR from mouse embryonic stem cell cDNA. For construction of an *Hmgpi* expression vector, the *Hmgpi* ORF was digested by *AgeI* and *BamHI*, and the fragment was cloned into the pAcGFP1-C1 vector plasmid (Clontech). The plasmid, digested by *MluI*, was used as template for *in vitro* transcription. RNA synthesis and poly(A) tailing were performed with a MEGAscript T7 kit (Invitrogen). Approximately 5–10 pl of 100 ng/µl *Hmgpi* mRNA in DEPC water (Invitrogen) was microinjected into the embryonic cytoplasm just after microinjection of si*Chd1*. The primers used for cloning are listed in supplementary material Table S2.

Statistical analysis

Each experiment was repeated at least three times. All data are expressed as means±s.e.m. Statistical analysis of the data was performed by analysis of variance (ANOVA) with Student's *t*-test. *P*-values <0.05 were considered to be statistically significant.

Ethical approval for the use of animals

All animal experiments were approved by the Animal Research Committee of Kyoto University (Permit number: 24-17) and performed in accordance with the guidelines of the committee.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

The experiments were designed by N.M., and were performed by S.S. and Y.N., with contributions from S.T., T.K. and I.M. The manuscript was written by S.S. and N.M., with contributions from H.I. All authors read and approved the final manuscript.

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Supplementary material

Supplementary material available online at
<http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.120493/-/DC1>

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